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Review

Rebuilding cancer metastasis in the mouse

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ABSTRACT

Most cancer deaths are due to the systemic dissemination of cancer cells and the formation of secondary tumors (metastasis) in distant organs. Recent years have brought impressive progress in metastasis research, yet we still lack sufficient insights into how cancer cells migrate out of primary tumors and invade into neighboring tissue, intravasate into the blood or the lymphatic circulation, survive in the blood stream, and target specific organs to initiate metastatic outgrowth. While a large number of cellular and animal models of cancer have been crucial in delineating the molecular mechanisms underlying tumor initiation and progression, experimental models that faithfully recapitulate the multiple stages of metastatic disease are still scarce. The advent of sophisticated genetic engineering in mice, in particular the ability to manipulate gene expression in specific tissue and at desired time points at will, have allowed to rebuild the metastatic process in mice. Here, we describe a selection of cellular experimental systems, tumor transplantation mouse models and genetically engineered mouse models that are used for monitoring specific processes involved in metastasis, such as cell migration and invasion, and for investigating the full metastatic process. Such models not only aid in deciphering the pathomechanisms of metastasis, but are also instrumental for the preclinical testing of anti-metastatic therapies and further refinement and generation of improved models.

1. Introduction

The most unfathomable component in tumorigenesis is the development of metastasis which accounts for more than 90% of cancer-related mortality and morbidity (Chaffer and Weinberg, 2011). Metastasis literally means "beyond stillness" and is the spread of cancer cells from the site of a primary tumor to distant anatomical sites within the body (Shibue and Weinberg, 2011). The genesis of metastasis has often been debated. In the linear progression model, one school of thought proposes that metastatic potential is a property acquired during the later stages of tumor progression by a few cancer cells due to accumulation of genetic alterations (Klein, 2009). The TNM classification of cancers (**T** describes the size of the **tumor**; **N** describes regional lymph **nodes** that are involved; **M** describes distant **metastasis**) based on the association of tumor size with increased metastasis derives from this model. In the parallel progression model, another school of thought argues that tumor cells may disseminate very early in malignant progression, colonize multiple secondary sites at different times and ultimately accumulate genetic changes independently from

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those incurred by the primary tumor (Sethi and Kang, 2011). Another interesting debate has revolved around explaining how and why cancer cells disseminate and colonize where they do. In 1889, Stephen Paget proposed the "seed and soil hypothesis" to explain the process of cancer cell dissemination (Paget, 1989). He posited that although tumor cells (seeds) are broadly disseminated during the course of malignant progression, detectable metastases only develop at those sites (soil) where the tumor cells are suitably adapted for survival and proliferation (Fidler, 2003; Fidler and Kripke, 1977). This hypothesis was challenged by Ewing in 1928 when he proposed that the anatomical layout and the circulatory patterns of the vasculature are accountable for the clinically observed occurrence of overt metastasis formation (Ewing, 1928). Both theories have stood the test of time and have been shown to contribute to the organ-specific tropism of cancer cell dissemination.

Extensive research has gone into understanding the nature and mechanisms of the 'black box' of metastasis and it has been only in the last decade that the molecular and cell-biological details of the mechanisms underlying this process have started to emerge. Also, an effective cure to curb cancer metastasis is still being explored. Hence, to be able to design effective cancer therapeutics, it is essential to model multistage carcinogenesis, including metastasis, in experimental systems. A major contributor that has helped to tease out the different facets of multistage tumorigenesis and metastasis has been the development of *in vivo* animal models that faithfully recapitulate the various stages of malignant cancer. In this review, we pay particular attention to the use and development of mouse models of metastasis that recapitulate malignant tumor progression as observed in patients and that have helped to delineate some of the molecular mechanisms underlying late stage cancer progression and metastasis.

2. Malignant tumor progression and metastasis

2.1. *Invasion-metastasis cascade*

Development and malignant progression of cancer is a multistage process. In particular, the metastases spawned by carcinomas are formed following the completion of a complex succession of cell-biological events collectively termed the invasion-metastasis cascade (Berx et al., 2007; Shibue and Weinberg, 2011). During this process, epithelial cells in the primary tumors undergo an epithelial–mesenchymal transition (EMT) and gain mesenchymal characteristics. They become migratory and invade locally through the surrounding extracellular matrix (ECM) and stromal cell layers by production of matrix metalloproteinases and other proteases. Cells eventually intravasate into the nearby blood and lymphatic vessels and then disseminate through the circulation. At a distant site, the cells may get trapped and extravasate into the tissue of a distant organ. If the secondary site is conducive, the cancer cells will generate micrometastasis and ultimately proliferate to generate macroscopic lesions (Figure 1). The multiple steps in the invasion-metastasis cascade are coordinated by molecular pathways operating within carcinoma cells as well as by heterotypic interactions between the carcinoma cells and the surrounding stromal cells, immune cells and extracellular matrix (Scheel et al., 2007).

Gene expression profiling studies with various cancer types have revealed the existence of pre-determining gene expression signatures that can predict the risk of metastatic recurrence. For instance, a set of 70 "poor prognosis gene signature" has been identified for breast cancer metastasis (van 't Veer et al., 2002; van de Vijver et al., 2002). This signature encompasses genes regulating cell cycle, invasion, metastasis and angiogenesis and is a powerful predictor of disease outcome in young patients (van de Vijver et al., 2002). In an independent study, the gene expression profiles of metastases of multiple cancer types have been compared to unmatched primary adenocarcinomas, revealing a 128 gene metastasis signature that distinguishes primary from metastatic adenocarcinomas (Ramaswamy et al., 2003).

2.2. *Metastatic organ tropism and the pre-metastatic niche*

An interesting aspect of metastasis is the proclivity of the cells of a particular cancer type to preferentially metastasize to certain organs. For instance, while breast cancer cells almost always spread to the bone, lung, brain and liver, prostrate cancer cells predominantly metastasize to the bone (Chiang and Massague, 2008). The influence of the circulatory patterns in the body and Paget's seed and soil hypothesis together may partially explain this biased organ tropism (Ewing, 1928; Paget, 1989). Yet, recent studies have led to the identification of gene signatures that define organ-specific cancer cell metastasis (Chiang and Massague, 2008; Nguyen and Massague, 2007). For instance, experiments in xenograft transplantation mouse models have identified and validated unique sets of genes that specifically promote metastasis of breast cancer cells to the bone, lung or brain (see below; Bos et al., 2009; Kang et al., 2003; Minn et al., 2005).

While the genetic and phenotypic makeup of cancer cells themselves has been conclusively shown to contribute to organ-specific metastasis, some recent elegant studies have also indicated a critical role of the tumor microenvironment in organ-specific cancer cell dissemination (Kaplan et al., 2006). For example, mouse transplantation models revealed that bone marrow-derived hematopoietic progenitor cells expressing vascular endothelial growth factor receptor-1 (VEGFR1) home to tumor type-specific "pre-metastatic sites" and form cellular clusters even before the arrival of tumor cells (Kaplan et al., 2005). In addition to specific gene signatures and the contribution of the microenvironment, metastasis of cancer cells is also influenced by activated tumor angiogenesis, by the immune system, and by epigenetic and genetic variations or polymorphisms in the cancer cell genome (Hanahan and Weinberg, 2000, 2011).

2.3. *Cellular experimental models of metastasis*

Over the years, there have been many attempts to rebuild the multiple stages of metastasis in culture systems *in vitro* and in animal models *in vivo*. These endeavors have been hampered mainly by a failure to recapitulate all the successive stages of malignant tumor progression and metastasis in one

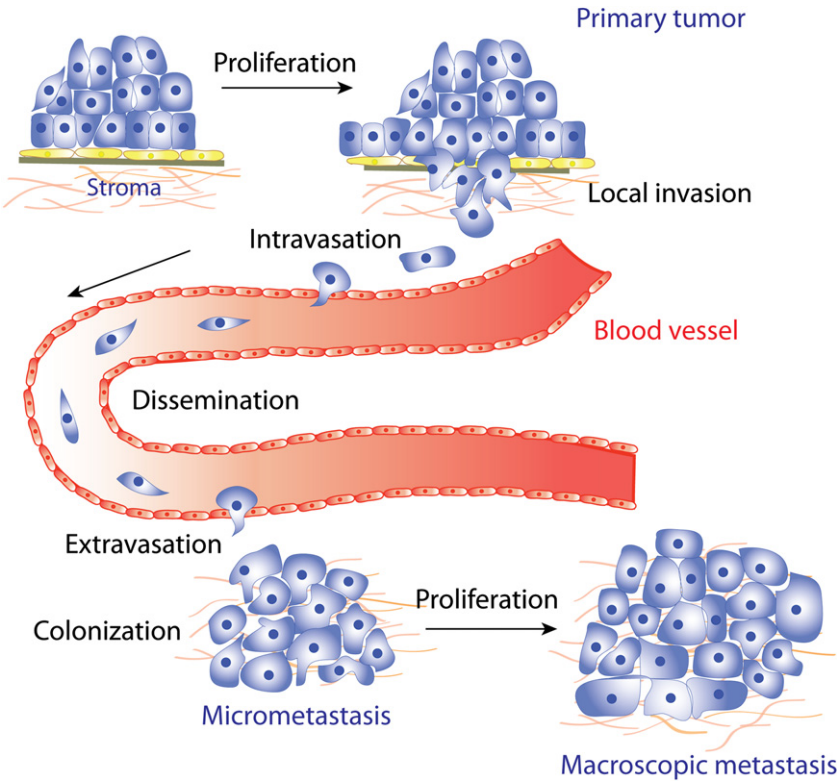

Figure 1 – Schematic representation of the multiple stages of metastatic dissemination of cancer cells from the primary tumor into the blood circulation, extravasation at a distant secondary site, colonization of a distant organ as micrometastasis and, finally, outgrowth as macroscopic metastasis.

experimental model. Hence, a number of 'reductionist approach' experimental systems have been established that mimic specific processes of the complex cascade of events during metastasis, such as cell migration, cell invasion or organ colonization. Here we describe the most relevant and frequently used experimental assays.

The hallmark properties of a metastatic cell are its ability to migrate and invade into the surrounding tissue (Zeisberg and Neilson, 2009). Initially, the migratory ability of a cancer cell has been tested in scatter assays. Cultured epithelial cells exhibit a cobblestone-like morphology and due to their strong cell–cell adhesions are essentially inert. Malignant cancer cells, however, gain the ability to migrate away from compact cell colonies leading to the phenomenon of cell scattering. Scatter assays have been used to demonstrate the phenomenon of EMT and the migratory effect bestowed by hepatocyte growth factor (HGF) on cancer cells (Stoker and Perryman, 1985). Another commonly employed *in vitro* assay for cell migration is the so-called scratch wound assay: a scratch is made into a confluent layer of cells in a tissue culture plate (Nobes and Hall, 1999). If the cancer cells are indeed migratory, they migrate into the gap to "close or heal the wound". In a more quantifiable assay, the migration of cells through a membrane in a Boyden Chamber is determined (Boyden, 1962; Reiske et al., 1999). Besides cell migration, an important feature of metastatic cancer cells is their ability to invade into the surrounding stroma by the secretion of proteases. The invasive capability of cancer cells can be assessed in a Matrigel invasion assay in which cancer cells are grown in chambers that contain a Matrigel-coated (extracellular matrix isolated from a mouse sarcoma cell line) membrane (Shaw, 2005). If cancer cells are indeed invasive, they degrade the Matrigel and invade through it to the other side of the filter where they can be stained and quantified. In a similar approach, the ability of cancer cells to invade through specific extracellular matrix components such as fibronectin or collagen can be measured (McClatchey, 1999).

Since the manifestation of metastasis is also affected by the interactions of the cancer cells with the tumor microenvironment, stromal cells, infiltrating cells of the immune system and blood vessels, there is a need for more appropriate experimental systems to mimic such complex interactions. One experimental system employs the chorioallantoic membrane (CAM) of the chick embryo. The CAM is rich in blood vessels, is composed of a multilayer epithelium, and contains extracellular matrix proteins, such as fibronectin, laminin and collagen type I. Since the lymphoid system is not fully developed until late stages of incubation, the chick embryo serves as a naturally immune-deficient host capable of sustaining xenografted tissues. Implantation of cancer cells into the CAM allows the live monitoring and also pharmacological manipulation of cell migration, cell invasion and the interaction of cancer cells with blood vessels. Although the CAM assay is mainly used as an angiogenesis assay, it has also served as an adequate experimental system to assess the invasion of various cancer cell types (Lokman et al., 2012).

3. Mouse models of metastasis

While *in vitro* model systems are instrumental in analyzing individual functional aspects of metastasizing cancer cells, the information pertaining to the entire complex process of metastasis is limited. Metastasis of cancer cells is the result of complex events within the cancer cells and of an intricate dialog with the tumor stroma. Hence, *in vivo* experimental systems that allow the investigation of cancer cell metastatic spread have been and are still being developed. Ideally, the use of human patient biopsies would enable a realistic and useful characterization of human cancer progression. However, the accessibility of tumor biopsies from all tumor stages in patients is limited; preneoplastic lesions usually remain undetected and metastases in many organs are difficult to biopsy. In contrast, animal models of tumorigenesis allow the reproducible isolation of all tumor stages, which are then amenable to pathological, genetic and biochemical analysis and manipulation. Furthermore, animal models that recapitulate the metastatic disease can serve as important preclinical tools for the testing and validation of novel cancer therapies in an effort to combat deadly metastasis. Thus, although animal models and patients are not physiologically identical, the use of animal models is the next to best option at hand to conduct meaningful experimentation. Animal models predominantly involve the transplantation of tumor cells into animals or the generation of genetically engineered animals that recapitulate the multistage process of metastasis. These are discussed in detail below.

Several animal models are being used in metastasis research, including the fruitfly drosophila, zebrafish, mice, rats and, more rarely, rabbits, companion pets, and monkeys (Berghmans et al., 2005; Blouin et al., 2005; Hubbard et al., 1984; Khanna and Hunter, 2005; Niu et al., 2012; Willecke et al., 2011). There are potential advantages and disadvantages to all. The most commonly used model species for deciphering malignant cancer has been the laboratory mouse which has proven to serve as a useful surrogate system to rebuild human cancer. Besides the anatomical, physiological and genetic similarities to humans, reasons for the use of mice include their small size, the ease of breeding and maintenance and their relatively short gestation time (Frese and Tuveson, 2007; Maddison and Clarke, 2005). The availability of the mouse genome and the ability to genetically manipulate mice by transgenic expression or by genetic knockout of genes of interest in a temporal and tissue-specific manner render mice very attractive for studying human cancer pathogenesis. As we describe below, with the technological advances in the past decades, mouse models have also evolved through several phases of increasing complexity, including xenograft tumors derived from tumor cell lines or explants, chemically or virally induced mutagenic models, and many variations of genetically engineered mice (GEM).

3.1. *Transplantation models*

Many important new insights into the pathomechanisms of cancer have been obtained by transplanting cancer cells into the mouse. Transplantation models have been basic tools to delineate the various stages of the invasion-metastasis cascade and also to develop, test and validate first therapeutic approaches.

3.1.1. *Syngeneic transplantation models*

Syngeneic transplantation refers to the inoculation of murine cells into another mouse recipient of the same genetic background (Fantozzi and Christofori, 2006; Khanna and Hunter, 2005). Syngeneic cell lines are either derived from spontaneously developing tumors or from carcinogen-, transgene- or gene knockout-induced tumors (Khanna and Hunter, 2005). Since the transplanted cells or tissue and the host are immune-compatible, the graft is not rejected (Fantozzi and Christofori, 2006). In the presence of a fully functional immune system, the contribution of the tumor microenvironment including host immune responses to tumor development and metastatic progression can be assessed. On the other hand, syngeneic models are based on inbred mouse strains and therefore the host organism lacks the genetic heterogeneity of human patients (Fantozzi and Christofori, 2006; Khanna and Hunter, 2005).

Serial transplantation of murine cell lines in murine hosts has been used to select cell line variants with higher tumorigenicity or metastatic abilities. For example, murine 4T1 mouse mammary carcinoma cells derived from a spontaneous Balb/c mouse mammary tumor are a commonly employed model of breast cancer metastasis. By serial transplantation of 4T1 cells in Balb/c mice, several sublines with distinct metastatic abilities have been generated (Lelekakis et al., 1999). Syngeneic transplantation of a 4T1.2 clonal variant into the mammary fat pad leads to a significantly higher incidence of bone, lung, and lymph node metastases as compared to parental 4T1 cells (Eckhardt et al., 2005; Lelekakis et al., 1999). Furthermore, the various sublines of 4T1 have also been used to generate distinct gene expression signatures for each stage of tumor progression, such as primary tumor formation, lymph node colonization, metastatic outgrowth in the lymph node, and distant organ metastasis. Comparison of the gene expression profiles of these sublines has led to the identification of Twist, an EMT-inducing transcription factor, which has also been shown to be important for breast cancer metastasis (Yang et al., 2004). Other popular models of syngeneic transplantation include the B16 melanoma model that closely mimics clinical melanoma metastasis and is available as sublines with various metastatic capabilities (Bobek et al., 2010).

3.1.2. *Xenotransplantation models*

Xenograft transplantation refers to the inoculation of (mainly) human cells or tissues into an immuno-compromised murine host (Fantozzi and Christofori, 2006; Khanna and Hunter, 2005). The major advantage of this system is the use of human tissue which enables modeling of human cancer metastasis in the mouse (Jonkers and Derksen, 2007). However, to prevent an immune rejection of human cells, the murine host needs to be devoid of a functional immune system. A variety of immune-compromised murine hosts for xenografting have been developed over the years, including Nude (nu/nu), SCID, SCID-Beige, NOD, NOD/SCID and NSG mice. These mice carry different gene mutations and thus exhibit various levels of immuno-deficiency (Table 1).

Table 1 – Immuno-compromised mouse lines used for xenotransplantation.

Name	Mutation in gene	Effects of mutation	Characteristics of mouse
Nude (nu/nu)	Foxn1^{nu}	Mutation affects the growth and differentiation of thymic epithelial cells leading to athymia. The mutation also leads to hairlessness.	T cell^{-}; B cell^{+}; NK cell^{+}; hemolytic complement^{+}
RAG$^{-/-}$	Genetic knockout of RAG1 or RAG2	Deficiency in RAG1 or RAG2 proteins which are responsible for activation of V(D)J recombination in T and B cells.	T cell^{-}; B cell^{-}; NK cell^{+}
SCID	Prkdc^{scid}	Mutation affects the protein that resolves DNA strand breaks during V(D)J recombination in T and B cells.	T cell^{-}; B cell^{-}; NK cell^{+}; hemolytic complement^{+}
SCID-Beige	Prkdc^{scid} and Lyst^{bg-J}	The SCID mutation affects the B and T lymphocytes. The beige mutation results in defective NK cells.	T cell^{-}; B cell^{-}; NK cells impaired
NOD-SCID	Prkdc^{scid} mutation in NOD background	SCID mutation on NOD background eliminates adaptive immunity and also reduces age-associated leakiness.	T cell^{-}; B cell^{-}; NK cell^{+}; hemolytic complement^{-}
NOG or NSG	NOD/ShiLtJ background, Prkdc^{scid} mutation, and an IL2 receptor gamma chain deficiency.	Combines the features of the NOD/ShiLtJ background, the Prkdc^{scid} mutation and an IL2 receptor common gamma chain deficiency which disables cytokine signaling and blocks NK cell differentiation.	T cell^{-}; B cell^{-}; NK cell^{-}; hemolytic complement^{-}; reduced dendritic cell function; reduced macrophage function; deficient in multiple cytokine signaling pathways

Abbreviations: SCID: severe combined immunodeficient, NOD: non-obese diabetic, NSG: NOD-SCID, common gamma receptor knockout, FOXN1: winged-helix/forkhead transcription factor, Prkdc: protein kinase, DNA-activated, catalytic polypeptide, RAG: recombination activating genes, NK cells: natural killer cells.

Due to the lack of a functional immune system in xenograft transplantation studies, the functional contribution of the immune system to metastatic progression cannot be assessed (Jonkers and Derksen, 2007). Yet, interactions between the tumor and the immune system have previously been shown to be important for tumor progression (Cabioglu et al., 2005; Orimo et al., 2005). Moreover, based on the species-specificity of some growth factor/growth factor receptor or cytokine/cytokine receptor systems, in xenograft models the relevance of paracrine interactions between tumor cells and tumor stroma is also limited. This problem can be at least partially overcome by humanizing the mouse recipient, for example by the co-implantation of human tumor cells with human stromal cells (Jonkers and Derksen, 2007; Kuperwasser et al., 2004, 2005).

3.1.3. *Transplantation models of metastatic dissemination*

Depending on the route of cancer cell delivery into the recipient mice, syngeneic and xenograft transplantation models of metastasis are further distinguished into experimental and spontaneous metastasis assays.

In experimental metastasis assays, the tumor cells (syngeneic or xenografts) are injected directly into the systemic circulation (Khanna and Hunter, 2005). The development of metastasis in these models is rapid and is largely influenced by the site of injection and the inherent tropism of the tumor cells (Khanna and Hunter, 2005). The various routes include lateral tail vein, intra-portal, intra-splenic, intra-carotid, intra-peritoneal and intra-cardiac injections (Khanna and Hunter, 2005). Injection into the tail vein is the most commonly used assay and primarily leads to formation of metastatic nodules in the lung (Arguello et al., 1988; Khanna and Hunter, 2005). Intra-portal vein and intra-splenic injection usually results in liver metastasis, intra-carotid injection leads to brain metastasis, and intra-peritoneal injection usually provokes local invasion (Khanna and Hunter, 2005; Lorger and Felding-Habermann, 2010). Intra-cardiac injection introduces cancer cells in the arterial circulation and result in metastases to several organs, including the liver, ovaries, adrenal gland, bone and brain (Arguello et al., 1988; Harms and Welch, 2003; Khanna and Hunter, 2005; Ottewell et al., 2006). Since the cancer cells are introduced directly into the circulation, the experimental models usually depict only the late phases of the invasion-metastasis cascade, such as cancer cell dissemination and organ colonization. Hence, a major drawback of this model system is that it fails to represent the earlier stages of the metastatic process, such as local invasion and intravasation, and it therefore should be considered as an assay of organ colonization and not of a true metastatic process.

In spontaneous metastasis assays, cancer tissue or tumor cells are primarily implanted into the organ from which the cancer cells have been originally derived (orthotopic transplantation) or into a tissue of high vascularization and convenient anatomical location that is not representing the organ of origin (ectopic transplantation), such as the skin or the subrenal capsule (Jin et al., 2010; Khanna and Hunter, 2005). Ectopic transplantation models mostly fail to mimic the appropriate microenvironment of the primary tumor and the corresponding metastatic dissemination to the relevant organs. Hence, orthotopic transplantation models have been developed that more closely mimic the human situation, including tumor histology, vascularity, tumor-stroma interactions, gene expression profiles, metastases development, and also chemotherapy responsiveness (Cespedes et al., 2006). With spontaneous models, most stages of the invasion-metastasis cascade can be observed and investigated. However, based on the multistage nature of the full metastatic

process, there is a high latency in the development of metastasis, and sometimes resection of the primary tumor is required to allow for the formation of metastasis (Banys et al., 2012; Francia et al., 2011).

The transplantable models primarily rely on the use of cancer cell lines which through continuous passaging in cell culture acquire notable as well as subtle changes that can significantly alter their properties (Fidler and Kripke, 1977). Moreover, although cell lines are heterogeneous, they may represent the expansion of a particular clone with high proliferative ability (Khanna and Hunter, 2005). Finally, the stroma associated with tumor cells plays an integral part in tumor progression. For instance, carcinoma-associated fibroblasts (CAFs) derived from a breast cancer patient support the growth of a breast carcinoma cell line in a xenograft mouse co-implantation model by activating the secretion of chemokines that can further recruit endothelial progenitor cells and promote tumor angiogenesis (Cabioglu et al., 2005; Orimo et al., 2005). Indeed, the grafting of fresh human tumor biopsies into immuno-compromised mice provides clinically relevant tumor models (Firestone, 2010; Jin et al., 2010; Rubio-Viqueira and Hidalgo, 2009). These systems have been used to personalize cancer treatment, as has been done for pancreatic cancers, and have also been used to study cancer-initiating cell populations by limiting dilution experiments and serial transplantation (Rubio-Viqueira and Hidalgo, 2009). For example, immuno-compromised NSG mice have been used to estimate the number of tumor-initiating cells by injection of patient-derived melanoma cells or even develop clinically relevant disease models, for example by serial transplantation of AML cells (Quintana et al., 2008; Sanchez et al., 2009). One limitation to the use of patient-derived xenografts is the low rate of their engraftment and tumorigenicity. However, co-injection along with stromal components like Matrigel or humanizing the recipient graft site with human stromal cells and matrix have been used to improve the outgrowth of xenografts (Kuperwasser et al., 2004, 2005).

3.1.4. *Organ tropism of metastasis*

The molecular basis for the specific organ tropism of cancer cells which leads to the formation of metastases in only particular organs has been an intense topic of research. The MDA-MB-231 invasive breast cancer cell line originally derived from a pleural effusion of a breast cancer patient (Cailleau et al., 1974), has played a central role in these studies. Upon orthotopic and subcutaneous transplantation into mice, this cell line efficiently forms not only primary tumors but also displays colonization of multiple target organs, such as liver, lung, brain, adrenal glands and bone (Cailleau et al., 1974; Price et al., 1990). In their seminal studies, the group of J. Massague serially transplanted MDA-MB-231 cells in nude mice to select for metastatic variants. While the consecutive intra-cardiac injections of these cells selected for sublines that preferentially metastasized to the bone or the brain, consecutive tail vein injections resulted into sublines that preferentially metastasized to the lungs (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005). Gene expression profiling of these sublines in comparison to the parental cells identified specific sets of genes that seemed to be important in dictating organ-specific tropism. For example, the bias for metastasis to the bone was found to be governed by genes like ADAMTS1, FGF5, FST, PRG, CTGF, IL11, MMP1 and CXCR4 (Kang et al., 2003). These genes play a role in angiogenesis, osteoclast differentiation, matrix degradation and the homing of cells to the bone, and hence vividly explain the inclination of these cells to metastasize to bone (Kang et al., 2003). In a similar manner, a 54 gene signature for lung-specific metastasis and 17 gene signature for brain-specific metastasis were obtained and functionally tested (Bos et al., 2009; Minn et al., 2005) (Figure 2). These studies in combination with the previous studies identifying metastasis-prognostic signatures have been particularly important as they challenge the linear progression model of metastasis and provided evidence for the presence of metastasis-competent cells even during the early stages of tumorigenesis (Ramaswamy et al., 2003; van 't Veer et al., 2002; van de Vijver et al., 2002). In similar studies, sublines of the breast cancer cell lines MDA-MB-435 and MDA-MB-468 have been generated that show differential colonization to specific organs, such as lung, lymph nodes, and thorax and have led to the identification of particular genes important for metastasis, such as CD73 and $\alpha 9\beta 1$ integrin (Lee et al., 2003; Vantyghem et al., 2005).

Several other noteworthy concepts regarding metastatic progression have also emerged from the use of transplantable models. These include the concept of a 'pre-metastatic niche', the first capillary bed encountered by cancer cells once introduced into the circulation or, in other words, the "congenial soil" or the microenvironment encountered subsequent to lodging in the target organ (Ewing, 1928; Kaplan et al., 2006, 2005; Nguyen and Massague, 2007; Paget, 1989). The existence of a pre-metastatic niche was first demonstrated in allograft transplantation models showing that bone marrow-derived hematopoietic progenitor cells expressing VEGF receptor-1 (VEGFR1+) home to tumor type-specific pre-metastatic sites and form cellular clusters even before the arrival of tumor cells (Kaplan et al., 2006, 2005). The VEGFR1-positive cells express integrin VLA-4, and the resident fibroblasts produce huge amounts of fibronectin, a VLA-4 ligand, providing a permissive niche for incoming tumor cells. Conversely, the absence of this pre-metastatic niche abrogates the development of metastasis (Kaplan et al., 2006, 2005).

3.2. *Genetically engineered mice (GEM)*

The transplantable models described above have undoubtedly provided a wealth of information regarding cancer development and progression. However, these models do not mimic all stages of metastasis progression and hence more refined models are needed for meaningful experimentation, for example by the use of genetically engineered mice (GEM).

The first generation of GEM reproducing multistage carcinogenesis included mice that had been engineered to express (transgene) or to lack (knockout) a gene of interest. Popular examples of transgenic mice are the MMTV-PyMT model of breast cancer (Guy et al., 1992; see also below) and the Rip1-Tag2 model of pancreatic β-cell carcinogenesis (Guy et al., 1992; Hanahan, 1985; Macleod and Jacks, 1999). First knockout mouse models of cancer were generated by targeting tumor suppressor genes of interest in embryonic stem cells (Macleod and Jacks, 1999). The first of such models included mice in which the Rb, Trp53 or NF1 tumor suppressor genes

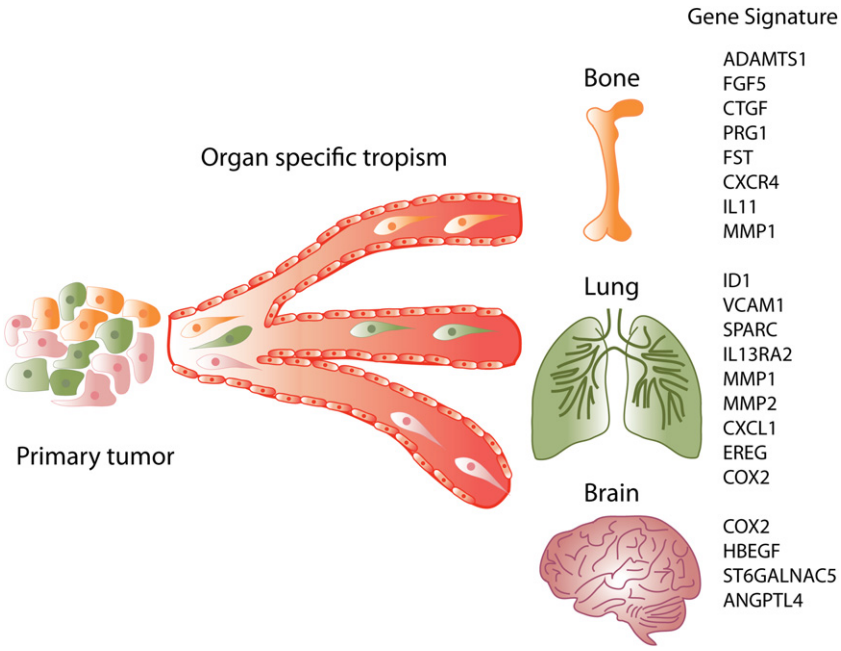

Figure 2 – Schematic representation of the organ tropism of cancer cells to various distant organs. Depicted are the formation of metastasis in bone, lung and brain and the gene signatures that have been associated with breast cancer cells metastasizing to the respective organs (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005).

were inactivated (Brannan et al., 1994; Donehower et al., 1992; Jacks et al., 1992, 1994a, 1994b; Lee et al., 1992). These first generation models have contributed significantly to the understanding of the molecular pathways responsible for the initiation and progression of human cancer and have highlighted the importance of specific oncogenes and tumor suppressor genes in carcinogenesis (Macleod and Jacks, 1999). Major advantages of these GEM models are the presence of an intact immune system and of species-specific tumor-stroma microenvironment interactions (Firestone, 2010). However, there remain some drawbacks with the use of these models. For instance, the constitutive expression or deletion of a particular gene can lead to embryonic lethality, severe developmental defects or sterility (Maddison and Clarke, 2005; Palmiter and Brinster, 1986). Further, the expression or disruption of the gene is systemic, i.e. in every cell of the organism or of a targeted organ, which mimics familial cancers but not the sporadic clonal development of most cancer types (Vignjevic et al., 2007). Most importantly, the first generation mice only rarely recapitulate all the stages of the metastatic cascade (Maddison and Clarke, 2005; Tuveson and Jacks, 2002).

With the advent of refined mouse genetic engineering technologies allowing the activation of transgenes and the inactivation of genes of interest in time and space and at will, new strategies were devised to generate models which mimic all stages of metastatic disease (Frese and Tuveson, 2007; Maddison and Clarke, 2005). However, even today there are only a handful of models that can recapture all the events of the invasion-metastasis cascade.

3.2.1. *Breast cancer models*

One of the most commonly employed models of metastatic breast cancer is the MMTV-PyMT model in which the mouse mammary tumor virus long terminal repeat (MMTV-LTR) has been employed to drive mammary gland-specific expression of the polyoma virus middle T antigen (PyMT) (Guy et al., 1992). The MMTV-PyMT model shares many aspects of human breast cancer progression, and multistage progression from hyperplasia to multifocal mammary adenocarcinomas followed by the development of metastatic lesions in lymph nodes and lung with high penetrance and short latency (Guy et al., 1992; Maglione et al., 2001). By crossing these mice with other GEM, the functional roles of various components of cellular signaling pathways, of the tumor microenvironment and of immune response have been addressed. For example, the importance of PI3K/Akt signaling in metastasis has been demonstrated in MMTV-PyMT; $Akt1^{-/-}$ mice (Maroulakou et al., 2007). The role of a chemoattractive paracrine loop of colony-stimulating factor-1 (CSF-1) and EGF ligands between tumor-associated macrophages (TAM) and tumor cells and its impact on invasiveness and lung metastasis has been shown in MMTV-PyMT; $Csf\text{-}1^{-/-}$ mice (Lin et al., 2001). The contribution of an innate and adaptive immune response to sustain metastasis has been revealed in MMTV-PyMT; $Rag1^{-/-}$ mice in which $CD4^{+}$ T cells are selectively lost or in MMTV-PyMT; $IL4^{-/-}$ mice in which interleukin-4 (IL4) is lacking (DeNardo et al., 2009). A critical role for the adhesion molecule CD44 in lung metastasis has been demonstrated in MMTV-PyMT; $CD44^{-/-}$ mice (Lopez et al., 2005). Finally, conditional ablation in MMTV-PyMT breast cancer cells has revealed pro-metastatic functions for the angiogenic factor VEGF-A and for the tumor progression factor TGFβ1 (Schoeffner et al., 2005; Muraoka-Cook et al., 2004). The metastatic incidence in MMTV-PyMT mice depends on the genetic background of the mice, suggesting that the polymorphic differences between the various inbred strains affects the incidence of metastasis

and that metastasis-modifier genes are waiting to be identified (Lifsted et al., 1998).

Another popular model expresses the oncogene ErbB2 (Her2/Neu) under the MMTV promoter in mammary epithelial cells (Muller et al., 1988). These mice develop multifocal adenocarcinomas with lung metastases at about 15 weeks after pregnancy. Expression of TGFβ in the breast cancer cells of MMTV-ErbB2; MMTV-TGFβ double-transgenic mice has been found to induce more circulating tumor cells and lung metastases than observed with MMTV-ErbB2 mice alone (Muraoka et al., 2003; Siegel et al., 2003).

Conditional deletion of tumor suppressor genes in mammary epithelial cells has also been successfully used for modeling metastatic breast cancer. MMTV-Cre; $Trp53^{fl/fl}$ mice develop breast tumors that efficiently metastasize to lung and liver (Lin et al., 2004). The latency period for primary tumor formation is even shorter in MMTV-Cre; $Brca1^{fl/fl}$; $Trp53^{+/-}$ mice, and metastases are detected in lymph nodes and lung (Brodie et al., 2001). Another model that develops metastatic mammary carcinoma resembling human invasive lobular breast cancer (ILC) has been generated by the concomitant ablation of E-cadherin and p53 expression in mammary epithelial cells (Derksen et al., 2006). This murine ILC model not only mimics human ILC, but also represents the first physiologically relevant model to study all aspects of breast cancer progression and metastasis (Derksen et al., 2006). However, thus far no efficient bone metastasis has been reported in GEM breast cancer mouse models.

GEM have also provided a wealth of information on the morphological and cellular processes in metastatic disease. For instance, MMTV-PyMT and MMTV-ErbB2 transgenic mice have been used to show that breast cancer cells leave the primary site even before the primary tumors become morphologically detectable (Husemann et al., 2008). Such disseminated tumor cells (DTCs) could be detected in the bone marrow and the lung. Another study has shown that premalignant cells also have the potential to disseminate and colonize a distant site. Untransformed mammary epithelial cells either from wild-type mice or from mice carrying inducible oncogenes are able to colonize a distant site (in this case, the lung) and to give rise to tumors at the distant site upon oncogene activation (Podsypanina et al., 2008). These studies support the parallel progression model of metastasis and argue against the hypothesis that metastasis can only evolve from a later, malignant stage of tumor progression.

3.2.2. *Prostate cancer models*

Several strategies have been employed to generate prostrate cancer mouse models (Ahmad et al., 2008; Rampetsreiter et al., 2011). For example, the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model for prostatic adenocarcinomas is based on the probasin (PB) promoter driving expression of SV40 T antigen in the prostatic epithelium (Gingrich et al., 1997; Greenberg et al., 1995). This model usually develops metastasis to the lung and lymph nodes but rarely to the bone, kidney and adrenal glands. The expression of the serine protease hepsin under the control of PB promoter in the LADY mouse model also generates metastasis (Klezovitch et al., 2004). In a knockin approach, the insertion of the gene encoding SV40 T antigen into the PSP94 (prostate secretory protein 94) genomic locus has produced mice (PSP-KIMAP) that develop prostatic intraepithelial neoplasia (PIN), invasive carcinomas and metastasis to the lymph nodes, lung and liver (Duan et al., 2005). The expression of SV40 T antigen under the control of Cryptdin-2 promoter has generated a model of neuroendocrine prostate cancer which metastasizes predominantly to lymph nodes and liver (infrequently also to the lung and bone) (Garabedian et al., 1998). Metastatic prostate mouse models have also been generated by the prostate-specific deletion of the tumor suppressor gene PTEN in ARR2PB-Cre; $PTEN^{fl/fl}$ composite mice. These mice develop metastasis to the lymph nodes and lung but not to bones (Wang et al., 2003). The conditional deletion of both PTEN and SMAD4 in the prostatic epithelium of compound ARR2PB-Cre; $PTEN^{fl/fl}$/$SMAD4^{fl/fl}$ mice generates prostate cancer with 100% penetrance of metastasis to lymph nodes and lung (Ding et al., 2011). Among other composite mouse models, $PTEN^{+/-}$; $Nkx3.1^{-/-}$ mice progress to invasive prostate cancer with lymph node metastases (Kim et al., 2002), while mice expressing FGF8b in $PTEN^{+/-}$ mice form adenocarcinoma and lymph node metastases (Zhong et al., 2006). Finally, conditional inactivation of Trp53 and Rb in the prostatic epithelium provokes prostate adenocarcinomas and metastasis to lymph nodes, liver and lung (Zhou et al., 2006).

3.2.3. *Lung cancer models*

In an effort to develop lung cancer models, researchers have used the surfactant protein C (SP-C) and the Clara cell secretory protein (CCSP) promoters for driving oncogene or Cre recombinase expression (Rampetsreiter et al., 2011). Expression of SV40 T antigen, c-Raf or c-Myc under control of these promoters results in aggressive adenocarcinomas (de Seranno and Meuwissen, 2010). Metastases are observed in SP-C-c-Myc single transgenic mice at lower frequency but combined expression of SP-C-c-Myc and SP-C-c-Raf in double-transgenic mice promotes the formation of metastases (Rapp et al., 2009). The most frequently used mouse model for inducible lung cancer development is the LSL-$KRAS^{G12D}$ model in which an activated form of KRAS is regulated by a LoxP-Stop-LoxP (LSL) transcriptional stop cassette located in front of the KRAS coding region (Guerra et al., 2003; Jackson et al., 2001). The administration of Cre recombinase by inhalation of recombinant adenovirus leads to the deletion of the LSL transcriptional stop cassette in front of the $KRAS^{G12D}$ transgene leading to its expression and the formation of lung adenocarcinomas (DuPage et al., 2009; Guerra et al., 2003; Jackson et al., 2001) or non small cell lung cancers (NSCLC) (Meuwissen et al., 2001). LSL-$KRAS^{G12D}$;$Trp53^{fl/fl}$ composite mice develop lung adenocarcinoma and metastases to lymph nodes, pleura, kidney, heart, adrenal glands and liver with high frequency (de Seranno and Meuwissen, 2010). Crosses of LSL-$KRAS^{G12D}$ with CCSP-Cre mice containing conditional alleles of LKB1, INK4a or Trp53 lead to the formation of metastasizing lung adenocarcinomas (DuPage et al., 2009). Finally, concomitant inactivation of the tumor suppressor genes Rb and Trp53 leads to metastatic small cell lung cancer (SCLC) closely resembling human SCLC (Meuwissen et al., 2001).

3.2.4. *Pancreatic cancer models*

Similar to lung adenocarcinoma models, many models for pancreatic ductal adenocarcinoma (PDAC) also rely on LSL-

$KRAS^{G12D}$ mice (DuPage et al., 2009). Deletion of the LSL transcriptional stop cassette in exocrine pancreas cells on exposure to Cre recombinase using Pdx1-Cre or p48-Cre transgenic mice results in the formation of PDAC and metastasis in the lymph nodes, liver, lung, pleura and the neural plexus (Grippo and Tuveson, 2010; Hingorani et al., 2003). In addition, compound Pdx1-Cre; LSL-$KRAS^{G12D}$ or p48-Cre; LSL-$KRAS^{G12D}$ mice with the additional deletion of the tumor suppressors INK4a, Trp53, SMAD4 or TGFβRII also develop invasive PDAC that metastasize to several organs (Aguirre et al., 2003; Grippo and Tuveson, 2010). Moreover, knockin of $KRAS^{G12D}$ into the Mist1 locus (Mist1tmKRASG12D mice), expressed during pancreas development, results in invasive and metastatic pancreatic cancers of different histopathologic appearance (Tuveson et al., 2006). Tumor progression and formation of liver metastasis are promoted in Mist1tmKRAS^{G12D}; Trp53$^{+/-}$ mice confirming the cooperativity between activated KRAS and the loss of Trp53 function (Tuveson et al., 2006). Another Cre-inducible pancreatic cancer model, EL-CreERT; cLGL-KRAS mice, also develop invasive and metastatic PDAC (Grippo and Tuveson, 2010).

3.2.5. *Colorectal cancer models*

Several important and useful models for studying colorectal cancer have been developed, for instance the APC^{min} model of intestinal adenoma, which however fail to progress to malignant cancer and metastasis (Jonkers and Berns, 2002; Rampetsreiter et al., 2011). Out of the many models available to study colorectal cancer, as of now only one model has been shown to robustly develop liver metastasis. LSL-$KRAS^{G12V}$; $APC^{fl/fl}$ mice combine the LSL-Ras^{G12V} Cre-inducible activated form of the Ras oncogene and the conditional deletion of APC in the intestine (Hung et al., 2010). Adenoviral administration of Cre into the colon leads to the loss of APC expression, the activation of $KRAS^{G12V}$ expression, and the generation of sporadic colorectal cancer that metastasizes to the liver (Hung et al., 2010).

4. Monitoring metastasis

With the need to generate refined mouse models that recapitulate the metastatic process, there also is a need to devise more sensitive and non-invasive strategies to visualize dynamic metastatic disease. Research over the recent years has brought forth many innovative strategies toward this aim. For instance, cancer imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and single photon emission computed tomography (SPECT) have been used extensively in basic research and in the clinic to monitor metastasis formation (Dubey et al., 2003; Naumov et al., 1999; Sweeney et al., 1999). Imaging modalities like CT and MRI provide a high degree of spatial resolution to study anatomical detail, while PET is sufficiently sensitive to monitor tumor burden, progression and metastasis. Bioluminescence imaging of tumor cells expressing firefly luciferase has allowed detecting macroscopic but not microscopic lesions in experimental models. Furthermore, the use of live animal fluorescent imaging techniques, in particular by the expression of genetically encoded fluorescence markers, have allowed to follow single metastatic cells in real time in experimental models *in vivo* and have improved our understanding of the biology of metastasis (Jenkins et al., 2003; Yang et al., 2000).

More recent developments, such as multimodality imaging technologies, amalgamate the properties of more than one imaging technique into a single experiment. One intriguing example is the use of a triple fusion protein reporter construct encoding for herpes simplex virus 1 thymidine kinase (HSV1-TK) fused to enhanced green fluorescent protein (eGFP) and to firefly luciferase (Ponomarev et al., 2004). Technological innovations, such as long-term spinning disk confocal microscopy and multiphoton laser scanning microscopy, have also enabled tracing of tumor cells and their interactions with the microenvironment (Carbonell et al., 2009; Egeblad et al., 2008). Intravital multiphoton (IVM) microscopy combines the advanced optical techniques of laser-scanning microscopy with long-wavelength multiphoton fluorescence excitation to capture high-resolution, three-dimensional (3D) images of living tissues that have been tagged with highly specific fluorophores (Condeelis and Segall, 2003). The use of IVM in animal models of cancer that express fluorescent proteins have enabled the direct visualization of cancer cells within a tumor *in vivo*. Both transplantable models and GEMs have been subjected to study by IVM and have elicited a number of differences between metastatic and non-metastatic tumor cells. For instance, metastatic cells show a linear and fiber-associated locomotion (Farina et al., 1998) and are more sensitive to chemotactic (or haptotactic) gradients (Wyckoff et al., 2000b). IVM has also demonstrated the contribution of macrophages and leucocytes to metastatic dissemination of cancer cells (Faust et al., 2000; Lin et al., 2001; Wyckoff et al., 2000a).

5. The ideal mouse model of cancer

Despite the progress that has been made in rebuilding cancer in mouse models, these models still suffer from a few flaws. For instance, GEM models develop tumors rapidly and grow to a size where they have to be surgically resected even if metastasis has not yet established (Politi and Pao, 2011). In addition, the relative penetrance for metastatic disease is often significantly lower than that of the primary tumor incidence (Cespedes et al., 2006). Furthermore, organ tropism in mouse models in most cases do not mimic the human situation (Rampetsreiter et al., 2011). Hence, there still is a need to develop the ideal mouse model for human cancer that faithfully recapitulates every feature of human metastatic disease. It should have similar histological and pathophysiological features, assemble the same genetic mutations and also progress through the same stages of the metastatic cascade as the corresponding human cancer. It should also respond to specific therapies in a manner comparable to the human counterpart. Finally, the full cancer phenotype should develop with high reproducibility and penetrance and in a short time frame. Such models would provide the required statistical power at low animal numbers to assess the functional contributions of modifier genes, of the tumor microenvironment and of immune cells to metastatic progression. This model would also allow the rapid and meaningful testing of potential therapeutic regimen.

Many argue that a mouse model can never fully recapitulate all aspects of human disease. Such belief stems from the fact that there are many inherent physiological differences between mouse and human (Khanna and Hunter, 2005; Rangarajan and Weinberg, 2003). To cite a few, mice have shorter lifespans, they exhibit long telomeres and constitutively active telomerase, and they differ in the composition of stromal elements, their xenobiotic metabolism, in their mutation rates and also transformation ability (Khanna and Hunter, 2005; Rangarajan and Weinberg, 2003). Also, most mouse models do not mimic human cancer metastatic progression. To overcome some of these limitations, multiple approaches have been taken to humanize mouse models. For example, implantation of human cancer-associated fibroblasts (CAF) into cleared mammary fat pads or grafting human bone pieces into mice recapitulates human metastatic breast cancer more closely (Kuperwasser et al., 2004, 2005). Mice can also be humanized genetically by replacing the relevant mouse genes with their human counterparts. For example, mice have been generated in which the human xenobiotic receptor XR or cytochrome P450CYP genes are expressed in mice that lack the corresponding murine genes (Gonzalez, 2004; Xie and Evans, 2002).

In summary, mouse models, be it transplantation models or GEM, have greatly contributed to our understanding of the basic biology of human cancer, the genes and pathways involved, and the role of cell non-autonomous components in metastatic progression. However, there are still major pitfalls for meaningful experimentation and inconsistencies between the models and human disease, and constant refinements and innovations are needed to generate more realistic mouse models of human disease. Since the ultimate goal of cancer research is to design effective therapeutic approaches to curb lethal metastatic disease, efforts need to be once more geared up and channeled toward the generation of the perfect surrogate mouse models of human cancer.

Conflict of interest

None declared.

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